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In vivo and *in vitro* analysis of electrical activity-dependent expression of muscle acetylcholine receptor genes using adenovirus

(E box/myogenic factor/muscle denervation/neuromuscular junction/gene transfer)

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ABSTRACT **Acetylcholine receptor (AChR) genes are repressed in extrajunctional domains of adult muscle fiber by neurally evoked electrical activity. Denervation elicits up-regulation of AChR gene transcription in extrasynaptic areas. We have used an adenovirus (Ad)-based strategy to analyze *in vitro* and *in vivo* the electrical activity-dependent transcription of the chicken AChR α_1 subunit gene. The luciferase gene placed under the control of wild-type and mutated fragments of the α_1 subunit promoter was inserted in a defective Ad vector designed for the study of transcriptional regulation. Animals were infected by intramuscular injection and *in vivo* luciferase levels were normalized by coinfection with an Ad vector containing the chloramphenicol acetyltransferase gene driven by an electrical activity-insensitive promoter. Our results demonstrate that although both proximal MyoD binding sites of the α_1 promoter are required for muscle-specific expression of the α_1 gene, only one is necessary, albeit insufficient, to enhance α_1 promoter activity after denervation. Parallel results were obtained with cultured muscle cells *in vitro* following tetrodotoxin blocking of spontaneous electrical activity. These results substantiate a direct contribution of MyoD factors in electrical activity-dependent regulation of AChR expression and further indicate that Ad-based vectors constitute a powerful tool in the field of transcriptional regulation.**

The nicotinic acetylcholine receptor (AChR) is a heteropentameric membrane protein whose distribution is restricted to the subsynaptic domain of the motor endplate in adult muscle fibers. This highly restricted topology results, at least in part, from the differential expression of the AChR subunit encoding genes by only the few subsynaptic nuclei, whereas in extrajunctional domains these genes are repressed by neurally evoked electrical activity (1, 2). Silencing of electrical activity by muscle denervation increases AChR expression in extrasynaptic muscle fiber nuclei. Run-on analysis (3) and transgenesis experiments (4–8) have demonstrated that repression of AChR expression by electrical activity occurs in part at the level of transcription of these genes.

Like many muscle-specific genes, the AChR promoters contain a binding site(s) for myogenic factors of the MyoD family that is necessary for muscle-specific expression (9–13). The members of this family (MyoD1, myogenin, Myf5, and MRF4) all contain the basic helix–loop–helix motif that is required for protein oligomerization and binding to the E-box DNA motif CANNTG (14). Since electrical activity modulates the expression of the myogenic factors themselves (15–18), they may be involved in electrical activity-dependent regulation of the AChR genes, although a direct role has not been yet demonstrated.

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It has been recently shown by transfection of primary cultures of rat myotubes that ≈180 bp of the mouse (17) and ≈100 bp of the rat δ-subunit promoter (19) contain electrical activity-responsive elements, but these elements, to our knowledge, have not been further characterized. Moreover, the physiological relevance of these primary culture data remains to be substantiated *in vivo*. On the other hand, studies using transgenic mice have shown that ≈850 bp of the α_1 (4, 7) and 2 kbp of the δ (8) subunit gene 5′ flanking sequences confer electrical activity-responsive expression to a reporter gene. To test for a direct contribution of the myogenic factors in the electrical activity-dependent expression of the AChR genes, we have exploited an alternative method based on recombinant human adenovirus (Ad) vectors that had been previously used to investigate the tissue-specific expression of different promoters in cell culture systems (20, 21). These linear double-stranded DNA viruses can infect post-mitotic cells, and, while this work was in progress, recombinant Ad vectors were reported to infect skeletal muscle (22, 23), thus allowing parallel *in vitro* and *in vivo* analysis of promoters.

By using Ad vectors, we have been able to characterize the differential contribution of two E boxes in the regulation of chicken AChR α_1 subunit encoding gene by electrical activity. Our results further demonstrate that myogenic factors are necessary, but insufficient, for this regulation.

MATERIAL AND METHODS

Plasmid and Virus Construction. For AdK8 construction, the +1 to +918 *Cla* I fragment of the human Ad5 genome that contains the Ad5 inverted terminal repeat was inserted in the *Cla* I site of pBR322 and further deleted to nt 128 using BAL-31 (giving rise to ITR 128 plasmid). A multiple cloning site fragment from pSP73 (Promega) and the +3328 to +6503 *Bgl* II–*Eag* I Ad5 fragment were subcloned into ITR 128 to generate the AdK8 cassette plasmid.

AChR α_1 subunit promoter fragments α842 [nt −842 to +20 (9)] and α110 [nt −110 to +1 (24)] have been subcloned into the *Bam*HI site of KS-ΔLA (24) to give rise to KS-842- and KS-110-ΔLA. The *Spe* I–*Kpn* I fragments of KS-842- and KS-110-ΔLA have been subsequently introduced in the *Xba* I–*Kpn* I AdK8 sites. Targeted mutagenesis of promoter fragments was performed as described (9).

The −256 to −62 promoter fragment of the chicken skeletal α-actin gene (25) was amplified from genomic DNA using

Abbreviations: AChR, acetylcholine receptor; Ad, adenovirus; TTX, tetrodotoxin; CAT, chloramphenicol acetyltransferase; pfu, plaque-forming unit(s); ITR, inverted terminal repeat.

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PCR, inserted in the KS Bluescript vector (Stratagene), and sequenced. The ASA promoter fragment was further placed upstream of the chloramphenicol acetyltransferase (CAT) sequence and subcloned into AdK8.

Recombinant viruses were obtained in 293 cells by homologous recombination between linearized Ad plasmid DNA and the right part of the In340 viral DNA as described (26), with few modifications: after *Cla* I–*Xba* I restriction, the right part of the viral DNA was purified on a NaCl gradient; 293 cells were transfected according to Chen and Okayama (27). For sequencing, low molecular weight DNA was prepared from 293 infected cells and PCR amplified (25 cycles) using AD1 (GTGATGTTGCCTGATCGATAAGC) and AD4 (CTCATCGTACCTCAGCACCTTCCA) primers. The amplification product was further sequenced without subcloning using an internal primer. Ad stocks were purified in CsCl gradients, dialyzed against a storage buffer (135 mM NaCl/1 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4/10% glycerol), and kept as frozen aliquots. Titers of the viral stocks were determined according to their optical densities (28), assuming that 1 OD unit corresponds to 1.1×10^{11} plaque-forming units (pfu).

Infection of Muscle Cell Cultures. Primary cultures of embryonic chicken myotubes were prepared as described (29). Before infection, cells were rinsed with prewarmed minimal essential medium (MEM) without serum; 3×10^8 pfu were diluted in 500 μl of MEM, spread on a 6-cm dish containing $\approx 5 \times 10^6$ cells, and incubated for 3 hr before adding fresh medium. Cells were harvested in a glycylglycine buffer (30) and lysed by three freeze/thaw cycles. Luciferase activity was quantitated as described (30).

RNase Protection. The αluc-RP plasmid was obtained after a *Spl* I–*Kpn* I deletion of KS-α842-luc (see above). After *Pvu* II digestion of αluc-RP, a ^{32}P-labeled riboprobe was synthesized using the T3 polymerase (Promega). Total RNA was prepared according to Chomczynski and Sacchi (31) from $\approx 2.5 \times 10^7$ cells infected by 6×10^9 pfu. RNase protection experiments were performed according to standard procedures.

Denervation Experiments. For *in vivo* experiments, viral stocks were diluted in storage buffer without glycerol. Leg muscles from 3-day-old White Leghorn chickens were bilaterally injected into the posterior muscle group with 50 μl of a mixture containing 3×10^9 v-α-luc and 3×10^9 v-ASA-CAT pfu. Mice were infected by transcutaneous injection of the posterior part of each leg with 4×10^9 v-α-luc and 3×10^9 v-ASA-CAT pfu. Denervation was performed by sciatic nerve section as described (3, 15). Animals were sacrificed by ether overdose. The posterior muscle group from each leg was dissected and immediately frozen in liquid nitrogen. Tissues were homogenized in 25 mM glycylglycine, pH 7.8/15 mM $MgSO_4$/15% glycerol/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride using a Polytron. Crude extracts were centrifuged and the supernatants were then frozen. CAT and luciferase activities were measured as described (30, 32).

Gel Retardation Assays. Chicken muscles were denervated by sciatic nerve section 1 day after birth. To prepare nuclear extracts, nuclei were purified according to Hahn and Covault (33) except that Percoll gradients had a concentration of 22% (vol/vol). Nuclear proteins were further extracted as described (34). Gel shift assays were as in ref. 24. Oligonucleotides were ^{32}P-labeled using polynucleotide kinase. E_B = 5′-CGTGGAACAGGTGGTGTA-3′, E_P = 5′-GCGCCCTCAGCTGTCATG-3′ (upper strands), and mutB is the E_B oligonucleotide that bears the mutB mutation (Fig. 2).

RESULTS

Design of an Ad-Based Vector for Promoter Analysis. In most Ad constructs, nonviral sequences are inserted in the left part of the Ad genome, downstream of an ≈500-bp viral fragment that contains the left ITR and the encapsidation sequence. However, this fragment has been shown to contain enhancer sequences of the Ad E1a gene promoter (35, 36). Since we planned to investigate promoter regulation, it appeared necessary to minimize eventual interference between the viral genome and the exogenous DNA fragment. A cassette plasmid (AdK8) was constructed that contains the left Ad ITR and a 3.2-kbp Ad5 fragment (nt 3328–6503) spaced by a polylinker in which the sequences to be studied were subcloned (Fig. 1*a*). The recombinant vectors were obtained by *in vivo* recombination between the linearized plasmid DNA and the right part of the In340 strain genome, which possesses a redundant encapsidation sequence. In the recombinant viruses, the exogenous promoter under study was located at the left end of the Ad genome, downstream of 128 bp that do not contain regulatory sequences (Fig. 1*b*). Viruses were grown in 293 cells that complement the deleted viral E1 functions.

The stability of the constructs was analyzed by restriction mapping of the viral DNA and sequencing of the proximal regions of the exogenous promoter fragments. No point mutations were observed, even after several rounds of amplification. As shown below, transcription arising from an AChR α_1 subunit promoter fragment was correctly initiated and restricted to differentiated muscle cells. Such recombinant Ad vectors thus appear as sufficiently stable and "neutral" vectors to analyze promoter fragments.

Requirement of the Proximal E_B Box for Enhanced α_1 Subunit Gene Transcription Following *in Vitro* Blockade of Electrical Activity. The promoter of the gene coding the AChR α_1 subunit contains two E boxes in the −80/−110 region (called E_P and E_B, Fig. 2*b*) that have been shown to be

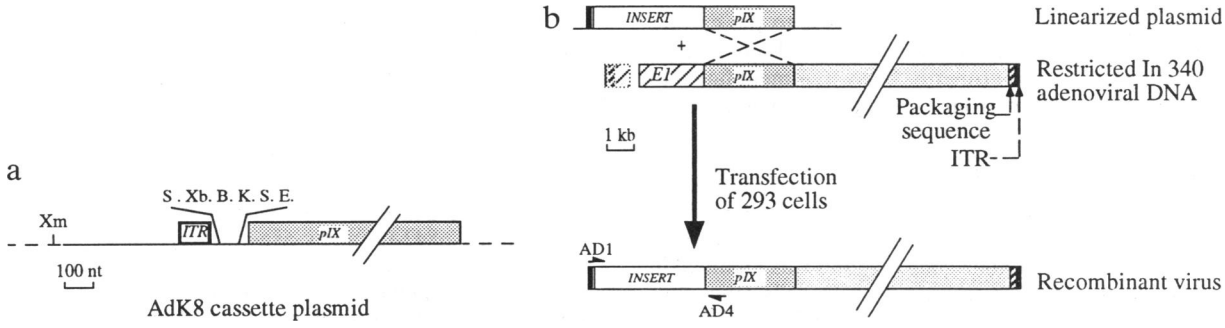

FIG. 1. (*a*) Schematic diagram of the AdK8 cassette plasmid. The white box corresponds to 128 bp from the Ad5 left terminus containing the ITR; the shaded box corresponds to the 3180-bp-long viral fragment containing the pIX viral peptide coding sequence; thin lines represent plasmidic sequences. S, *Sal* I; Xb, *Xba* I; B, *Bam*HI; K, *Kpn* I; E, *Eco*RV; Xm, *Xmn* I. (*b*) Virus construction strategy. The plasmid containing sequences to be inserted in the Ad is linearized and cotransfected in 293 cells with the purified right part of the In340 DNA. After homologous recombination between both DNA fragments, encapsidation sequence is located at the right extremity of the genome. Arrowheads represent the AD1 and AD4 primers that are used to amplify the fragments to be sequenced.

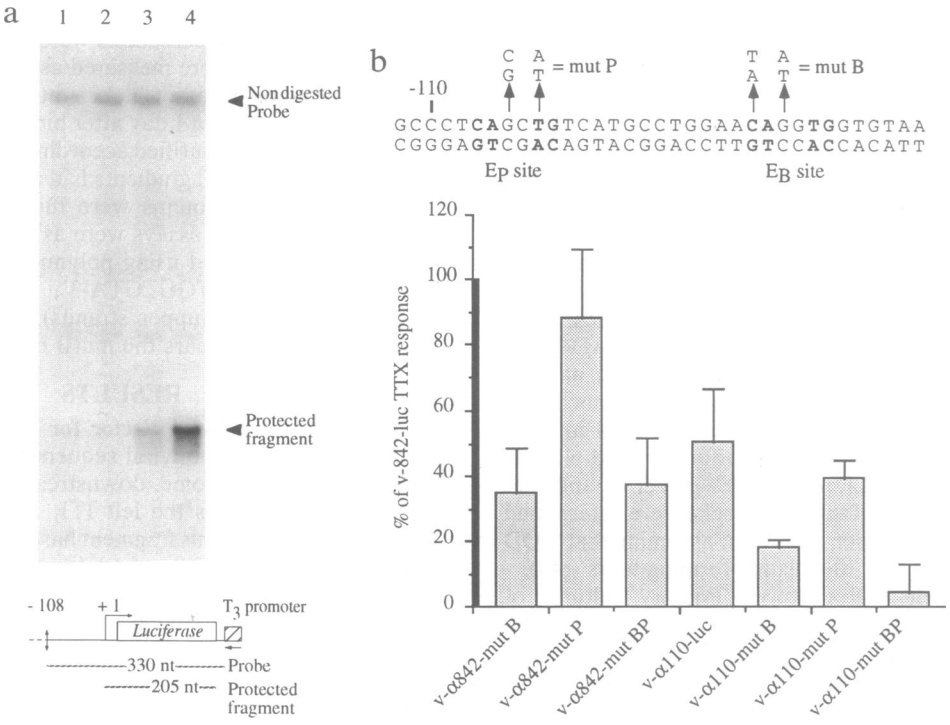

FIG. 2. (*a*) Characterization of the luciferase gene transcription start site in v-α842-luc-infected myotubes using RNase protection. Sixty micrograms of RNA was used in each lane. Lane 1, yeast tRNA; lane 2, total RNA of mock infected myotubes; lanes 3 and 4, total RNA of v-α842-luc-infected myotubes that were electrically active (lane 3) or inactive (lane 4). (*b*) Induction of luciferase expression after treatment of chicken myotube primary cultures with tetrodotoxin (TTX). The sequence corresponds to the fragment of the AChR α_1 subunit promoter that contains the E_P and E_B sites (CANNTG motifs are indicated in boldface type). Position −110 is relative to the transcription start site. TTX treatment of v-α842-luc-infected myotubes elicits a 2.0-fold [±0.3-fold (mean ± SD, n = 9 independent experiments)] increase in the luciferase activity level. To compare the results from experiments in which TTX effect varied, data are presented as percent of the v-α842-luc response. For each construct, average luciferase values were quantitated for triplicate cell dishes that had been either TTX treated (x_T) or nontreated (x_N); the fold induction of each construct was calculated as $(x_T - x_N)/x_N$ and further normalized to the v-α842-luc value. Data are presented as mean ± SD (n = 6–9 independent experiments), and at least two different viral stocks have been used for each construct.

necessary for muscle-specific expression of the α_1 subunit gene (9). Ad vectors containing the luciferase gene under the control of wild-type or E-box-mutated promoter sequences have been constructed: v-α842-luc ("wild-type") contains the −842 to +20 chicken α_1 subunit promoter fragment. Mutation of the E_B and E_P sites is called mut B and mut P, respectively (Fig. 2*b*). All constructs expressed luciferase at the same low levels in nonmuscle cells and in myoblasts. Luciferase expression levels arising from E-box-mutated promoter fragments were lower in differentiated muscle cells compared to the wild-type construct, in agreement with transfection experiments (9) (data not shown).

In primary cultures of chicken myotubes, myofibers exhibit spontaneous electrical activity, and its blocking by addition of the sodium channel blocker TTX to the culture results in a >10-fold increase in the AChR α_1 subunit mRNA levels (29). In previous experiments, TTX treatment failed to activate the transcription from α_1 subunit promoter fragments using various transfection methods. In contrast, TTX treatment of v-α842-luc-infected myotubes elicited a 2.0-fold [±0.3-fold (mean ± SD, n = 9 independent experiments)] increase in the luciferase activity level. Transcription of the luciferase gene was initiated at the same site of the α_1 subunit promoter as in the endogenous gene (37) and was identical in TTX-treated and non-TTX-treated muscle cells (Fig. 2*a*). Such an effect was of smaller amplitude than effects observed at the endogenous mRNA level. Nevertheless, run-on experiments have not resulted in the detection of more than a 2- to 3-fold increase in the rate of transcription of the α_1 subunit gene following TTX treatment of myotubes (A. Duclert and J.P., unpublished data). These results substantiate the notion that electrical activity represses expression of the α_1 subunit gene, at least in part, at the level of transcription. They further support the idea that Ad infection represents a more sensitive method than the standard calcium phosphate transfection techniques for the study of transcriptional regulation.

Subsequently, we examined the involvement of the E boxes in induction of α_1 subunit promoter activity by TTX (Fig. 2*b*). The 110-bp fragment remained TTX responsive, but the magnitude of the effect was 50% smaller than that observed with v-α842-luc. Mutation of the E_P site had no effect in either the −842 or the −110 context. In contrast, disruption of the E_B site, alone or in combination with the E_P site mutation, resulted in a 50% loss of responsiveness in the −842 context and a totally TTX-insensitive promoter when introduced in the context of the 110-bp fragment. These data demonstrate that (*i*) E_B and E_P sites are nonequivalent, (*ii*) E_B is a cis-acting element directly involved in the regulation of the AChR α_1 subunit gene expression by electrical activity, and (*iii*) E_B is necessary, but insufficient, to get a TTX-responsive promoter.

***In Vivo* Analysis of Electrical Activity-Dependent Regulation of the AChR α_1 Subunit Gene Using Ad Vectors.** The transcriptional regulation of the chicken α_1 gene was then examined *in vivo*. Chicken legs were bilaterally injected with a mixture of v-α842-luc and v-asa-cat, which bears the bacterial CAT gene under the control of the chicken skeletal α-actin promoter (fragment −256 to +62) (25). Five to 7 days after injection, one leg was denervated. Muscle extracts from innervated and denervated limbs were subsequently prepared to quantify CAT and luciferase activities. The ratio of CAT activity between innervated and denervated muscles was found to be 1 [0.98 ± 0.54 (mean ± SD, n = 73 animals)], in agreement with the stability of skeletal α-actin expression in recently denervated muscles (15). CAT activity was thus

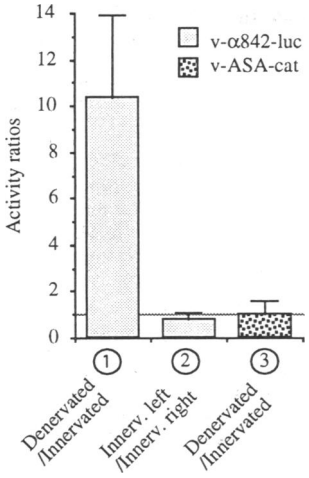

FIG. 3. Differential effect of muscle denervation on the activity of the AChR α_1 subunit and skeletal α-actin gene promoters. Leg muscles from 3-day-old White Leghorn chickens were bilaterally injected into the posterior muscle group with recombinant viruses. Five to 7 days later, the right or left sciatic nerve was sectioned. Animals were sacrificed after 3 days. Data are presented as the ratio of enzyme activity in the two legs of each individuals. Bars indicate the SD between different individuals from independent experiments (1: n = 14, 5 experiments; 2: n = 4, 2 experiments; 3: n = 73, 5 experiments). Luciferase values are normalized to CAT levels derived from v-ASA-CAT coinfection (v-ASA-CAT contains the CAT gene placed under the control of a promoter fragment of the chicken skeletal α-actin gene).

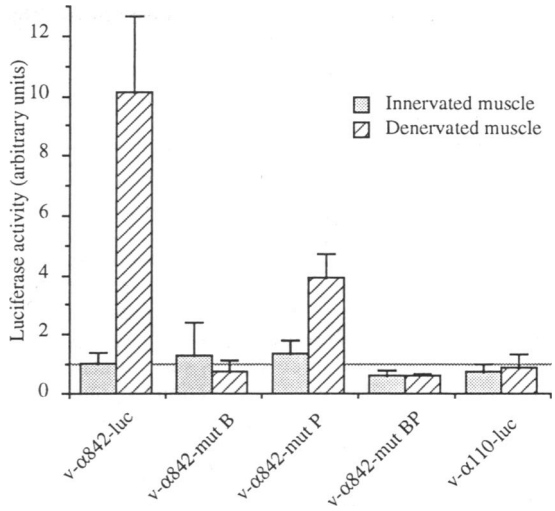

FIG. 4. Denervation experiments in the mouse. Five-week-old CBA mice were infected by transcutaneous injection of the posterior part of each leg with a v-α-luc and v-ASA-CAT mixture. Five to 7 days later, the right or left sciatic nerve was sectioned. Animals were sacrificed 50 hr after denervation. Luciferase activities were normalized to CAT levels and the values are presented in arbitrary units, where 1 = the activity in v-α842-luc-infected innervated muscles. Bars represent the SD between three individuals. The data presented are from one experiment representative of four.

taken as an index of infection efficiency and used to normalize the expression of the v-α842-luc construct. Three days after nerve section the luciferase levels were 10-fold higher in denervated versus innervated muscles (Fig. 3). These results are consistent with previous run-on studies that reported a 7-fold enhancement of the α_1 subunit gene transcription rate in chicken denervated muscle (3).

E_B Dependence of AChR α_1 Subunit Gene Expression in Denervated Adult Muscle. Ad vectors containing mutated promoter sequences were tested for their inducibility by denervation. Although wild-type and mutated fragments consistently gave different results in newborn chickens, variations between individuals were too large to allow a conclusive interpretation of the data (not shown). The rapid development of chicken muscle mass during the first postnatal weeks most likely explains these variations. Since experiments with adult hen muscle appeared technically difficult, we shifted to the adult mouse. In innervated mouse muscles, v-α842-mut B, -mut P, -mut BP, and v-α110-luc elicited luciferase expression levels not significantly different from those obtained after injection of the v-α842-luc vector. However, v-α842-mut B, -mut BP, and v-α110-luc did not respond to denervation, whereas v-α842-mut P luciferase expression reached one-third to one-half of v-α842-luc levels (Fig. 4). These data confirm that E_B and E_P sites are not equivalent and further demonstrate that (*i*) E_B and, to a lesser extent, E_P are required for α_1 subunit gene expression in denervated muscle and (*ii*) E boxes are necessary, but insufficient, to get denervation-induced transcriptional enhancement, since the α110 promoter fragment retains the two E_B and E_P sites but does not respond to denervation.

Different *in Vitro* Binding Affinities of E_B and E_P for Nuclear Factors. Incubation of the E_B or E_P site with nuclear extracts from chicken innervated muscle generated complexes whose electrophoretic mobility appeared identical in both cases (Fig. 5*a*). E-box-mutated oligonucleotides fail to competitively inhibit either the E_B or E_P complexes (Fig. 5*b*, lanes 6 and 7 and data not shown). These complexes thus represent specific interactions with the E motif. Using extracts from denervated muscles, only a weak increase in the abundance of the slower migrating complexes was noticed, a result in agreement with previous gel shift experiments using the IIb oligonucleotide that contained the E_B site (34). The retardation patterns of E_B and E_P remained identical, but the binding affinity of the nuclear proteins to E_B was roughly one order of magnitude greater than that of E_P on the basis of signal intensities (Fig. 5*a*, compare lanes 1–4 with lanes 5–8) and competition experiments (Fig. 5*b*, compare lanes 2 and 3 with lanes 4 and 5). A similar difference in binding affinity between E_B and E_P was found using a recombinant glutathione *S*-transferase–myogenin fusion protein (not shown).

DISCUSSION

Ad vectors have allowed a comparative and quantitative study of the transcriptional regulation of the AChR α_1 subunit encoding gene both *in vitro* and *in vivo*. Analysis of the promoter by deletion and site-directed mutagenesis in the Ad

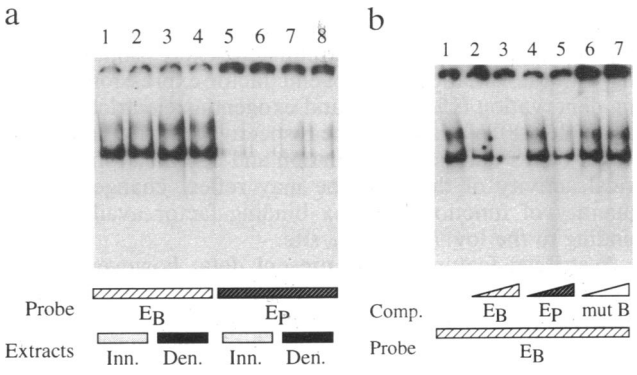

FIG. 5. Gel retardation assays using extracts of chicken muscle. (*a*) One nanogram of the E_B (lanes 1–4) or E_P (lanes 5–8) labeled probe was incubated with 1 μg of a nuclear extract from either innervated (Inn.) (lanes 1 and 2 and lanes 5 and 6) or 4-day denervated (Den.) (lanes 3 and 4 and lanes 7 and 8) chicken muscles. (*b*) As above using extracts from innervated muscles. Unlabeled competitor (Comp.) oligonucleotides were added in a 5- and 50-fold molar excess.

context confirms that both proximal E boxes of the chicken α_1 subunit promoter contribute to the muscle-specific expression of the gene, but only one of them, the E_B site, is significantly involved in the electrical activity-dependent regulation of α_1 subunit gene expression.

Ad-based vectors have presented a number of advantages over formerly used methods. The recombinant viruses are nonpathogenic, stable, and easily propagated, and viral stocks with high titers ($\approx 10^{11}$ pfu/ml) are routinely obtained. Viral sequences did not interfere significantly with the promoter fragment since α_1 subunit promoter-derived transcription was correctly initiated and muscle-specific expression was preserved. Infection of chicken myotubes with Ad-based vectors allowed the study of TTX effects on α_1 subunit gene transcription; this was not possible using transfection techniques in these cultures (see, however, refs. 38 and 17 with rat myotubes). Ad infection thus seems a more "physiological" method to introduce foreign DNA into cells. The main advantage of the Ad technique, however, is the possibility to investigate transcriptional regulation *in vivo*, with the same vector used *in vitro*. Studies can be performed in various species such as rat, chicken, or quail (unpublished data) in which transgenesis is not yet available. Most tissues, including brain (39), can be efficiently infected even during the adult stage. Ad remain extrachromosomal, hence, precluding genomic insertional influences (40). Moreover, since normalization strategies are available, it becomes possible to *quantitatively* analyze the activity of deleted or mutated promoter fragments *in vivo*. Ad vectors thus constitute a powerful addition to transgenesis experiments in the field of transcriptional regulation.

In light of the results obtained with the Ad-based method, the relative contribution of the E_P and E_B sites to the α_1 subunit gene transcription level varies according to the differentiation state and electrical status of the muscle fiber. In the few differentiated myotubes that do not yet contract, both E boxes are required for high expression levels, as previously shown by transfection experiments (9). In innervated muscle where AChR genes are repressed, the E boxes do not seem to contribute to the low luciferase expression levels that are detected after infection with v-α-luc vectors. In contrast, the E_B site is predominantly involved in the transcriptional enhancement that follows blockade of electrical activity, either after TTX treatment of spontaneously contracting myotubes or following muscle denervation. In gel shift experiments, the binding affinity of nuclear proteins to the E_P site is roughly one order of magnitude below that to the E_B site. No binding cooperativity between the two sites has been shown when using purified MyoD protein in gel retardation assays (9). These results show that E boxes directly participate in the muscle-specific *and* the electrical activity-dependent regulation of α_1 subunit gene expression, in keeping with the variation of myogenic factor expression following denervation (15, 16, 41) and exogenous electrical stimulation (16, 18). Variation of the respective contribution of the two E boxes during differentiation and modification of electrical activity of the myotube may reflect changes in the quantity of functional E box binding factor available for binding to the low-affinity E_P site.

A striking feature of the present data, however, is the requirement of an additional cis-regulatory element(s) that cooperates with the α_1 proximal E boxes to obtain an electrical activity-sensitive promoter. The interaction of myogenic factors with other regulatory proteins has been described for the α_1 subunit promoter (24) and other muscle-specific promoters (42, 43). Since many muscle-specific promoters that are not regulated by electrical activity do contain functional E boxes, it is tempting to speculate that myogenic factors combine with additional factors in a form that would be specific for electrical activity-regulated promoters in electrically inactive myotubes. On the other hand, they could cooperate with a regulatory protein(s) specifically expressed after electrical activity blockade. This is a challenging question that must be investigated further.

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